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THE PREFERENTIAL EXTRACTION OF KERATOHYALIN GRANULES AND INTERFILAMENTOUS SUBSTANCES OF THE HORNY CELL*

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Previously we demonstrated at least two sites for synthesis of different structural proteins in the epidermis, one in granular cells, the other in basal and Malpighian cells (1–4). As the cells moved outward these proteins appeared in the cornified layer, implying that both proteins contribute to form cornified cells. Further study using electron microscopic autoradiography showed that incorporation of histidine-H^3 into protein of granular cells resulted in formation and enlargement of keratohyalin granules (5). Incorporation of cystine-H^3, on the other hand, was found to contribute to the plasma membrane of cornified cells during the process of thickening (6). It appears, therefore, that a specific protein is formed during each step of the differentiation process leading to cell cornification. Chemical studies to date have been insufficient to elucidate the nature of these proteins, since different cellular components have not been isolated selectively.

We have attempted to preferentially extract epidermal cellular structures with 0.1 N NaOH treatment of glutaraldehyde fixed tissue, the rationale being that glutaraldehyde "fixes" different proteins in varying degrees by cross-linking polypeptide chains (7). The Schiff reaction was employed to demonstrate cellular sites fixed by glutaraldehyde since a positive reaction is induced in tissues by this fixative (8). Histidine-H^3 (or leucine-H^3) was injected intradermally in order to isotopically label protein synthesized in granular cells (or protein produced in the lower layers), and the amount of radioactivity solubilized from the epidermis by NaOH was counted. In addition, changes in distribution of grains in the epidermis before and after extraction were determined by means of autoradiography to elucidate the source of solubilized substances. Ultrastructure of the extracted tissue was examined for remaining cellular components.

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MATERIALS AND METHODS

Animals. The dorsal side of the skin from newborn rats of the Sprague-Dawley strain, 4–5 days old, was used in the study.

Glutaraldehyde fixation. Fifty per cent of glutaraldehyde solution (Fisher Lab.) diluted to 3 per cent with 0.05 M phosphate buffer containing 1 per cent sucrose, pH 7.4, served for fixation, and is referred to as glutaraldehyde. Tissues were fixed overnight at 4° C and subsequently were washed 3 times in 2 hours with phosphate buffered 1 per cent sucrose solution.

Schiff reaction on fixed tissue. Skin biopsies (0.5 x 0.5 x 0.1 cm) fixed in glutaraldehyde were dehydrated and embedded in paraffin. Sections cut at 4 μ were stained with Schiff reagent for 1 hour at room temperature followed by 3 washes of potassium metabisulfite solution.

Light and electron microscopic techniques. Biopsy specimens were diced and fixed in glutaraldehyde. Half of each specimen was incubated in 0.1 N NaOH at 37° C for 24 hours and separately post-fixed for 2 hours in 2 per cent osmium tetroxide buffered with phosphate at pH 7.4. After dehydration tissues were embedded in a mixture of Araldite and Epon and cut at 0.5 μ and 600–800 A. Thick sections were stained with toluidine blue and thin sections were stained with uranyl acetate and lead citrate. Some tissues were embedded in paraffin without post fixation in osmium tetroxide, cut at 4 μ and stained with hematoxylin and eosin.

Determination of soluble radioactivity in 0.1 N NaOH. Ten μc of histidine-H^3 (Spec. Act. 4.35 c/mM) or leucine-H^3 (Spec. Act. 2.0 c/mM) was injected intradermally in the animals. Biopsies (approximately 1.0 x 1.0 cm) secured at 30 minutes, 3 and 6 hours, were soaked for 10–15 minutes at 0° C in 0.24 M NH_4Cl, pH 9.5, to separate epidermis from dermis. Tissues were fixed in glutaraldehyde and incubated for 2, 6, and 24 hours in 0.1 N NaOH, at 37° C. Solubilized radioactivity was counted by a scintillation counter (Beckman LS-200). The insoluble epidermal residue was hydrolyzed in hot 6 N HCl for 24

hours and its radioactivity was counted for comparison with that found in NaOH solution.

Determination of protein in 0.1 N NaOH extracts. Presence of protein in extracts was determined by the biuret reaction (9).

Autoradiographic techniques. After injection of histidine-H^3 or leucine-H^3, as mentioned above, skin specimens were obtained at 30 minutes, 3 and 6 hours. They were diced and fixed in glutaraldehyde and prepared for 0.5 μ sections, as described in the section on Microscopic Techniques. A dipping method with NTB-2 emulsion was used for filming those sections mounted on microslides. After 3 weeks exposure at 4° C, the films were developed with Amidol developer and fixed with Edwal Quick Fixer. Specimens were stained with alkaline methylene blue solution. The number of grains appearing in each cell layer was counted and computed by the method described previously (2–4).

RESULTS

Localization of Schiff positive reaction. A positive reaction indicating sites of glutaraldehyde binding in the tissue was observed in the nuclei and cytoplasm of all viable epidermal cells, but not in keratohyalin granules. In the cornified layer, the lowermost 3–4 cells and the outermost 2–3 cell layers gave a positive reaction but the middle layers demonstrated a much weaker reaction.

Morphological changes in the epidermis after incubation with 0.1 N NaOH. Light microscopic findings—at 2 hours after incubation, reduction in the staining of keratohyalin granules and the cornified layer was observed in plastic embedded tissue (Figs. 1a and 1b). Sections embedded in paraffin and stained with hematoxylin and eosin indicated this change corresponds to loss of basophilic material of keratohyalin granules. The process was much more pronounced by 6 hours and was completed after 24 hours extraction; keratohyalin granules then appeared as clear spaces in the epidermis (Figs. 1c and 1d). Cornified cells in the outer two-third layers became empty and only cell outlines remained.

Electron microscopic observations—after 24 hours extraction, cornified cells no longer showed the typical organization of filaments in a dense matrix described as "keratin pattern" by Brody (10) (Fig. 2a). All filaments were well-stained. Often the filaments were bundled and attached to cell membranes whose two layers had become apparent (Fig. 2b). Although a densely stained intercellular contact layer was extracted, it seems reasonable to consider these attached areas are the attachment plaques of desmosomes observed in cells of the lower layers of the epidermis (Figs. 2b and 3). Cells located just above the granular layer that normally stain quite densely and show very little internal structure readily revealed their fibrillar structure after extraction; longitudinally cut fibers had the same appearance as tonofilament bundles in cells below (Fig. 4). In granular cells, keratohyalin granules lost densely stained material and a very fine fibrillar structure became visible (Figs. 4 and 5). Morphological features of cytoplasmic organelles such as mitochondria and polysomes were less defined, but fibrils and nuclei remained well-preserved in cells of all layers (Fig. 5). Attachment of tonofibrils to the desmosomes persisted, although the intercellular contact layer was dissolved (Figs. 4 and 5).

Determination of solubilized protein and radioactivity in 0.1 N NaOH solution. Presence of protein in the extract was identified in all specimens using the biuret reaction, but quantification was not carried out in this study. Table I summarizes the amount of radioactivity solubilized after different extraction times, in comparison with the total radioactivity present in the epidermis (sum of soluble and insoluble radioactivity), at various intervals after injection of leucine-H^3 and histidine-H^3. Since fixation and subsequent washing have been found to eliminate nonprotein radioactivity from epidermis (5), solubilized radioactivity in 0.1 N NaOH was considered to result from radioactively labeled protein. Large amounts of radioactivity were solubilized from tissue containing histidine-H^3 labeled protein, but only 5–7 per cent from tissue containing protein labeled with leucine-H^3.

Distribution of radioactive proteins before and after 0.1 N NaOH extraction. Radioactivity was distributed throughout all three viable layers of the epidermis 30 minutes after injection of leucine-H^3 and histidine-H^3. However, as we have reported previously (1–3), concentration of grains in the various layers of the epidermis differed significantly after injection of the two tritiated amino acids; grains were primarily located in the granular layer after injection of histidine-H^3 (Fig. 6a), whereas labels appeared more over the basal layer after injection of leucine-H^3. At 6 hours the

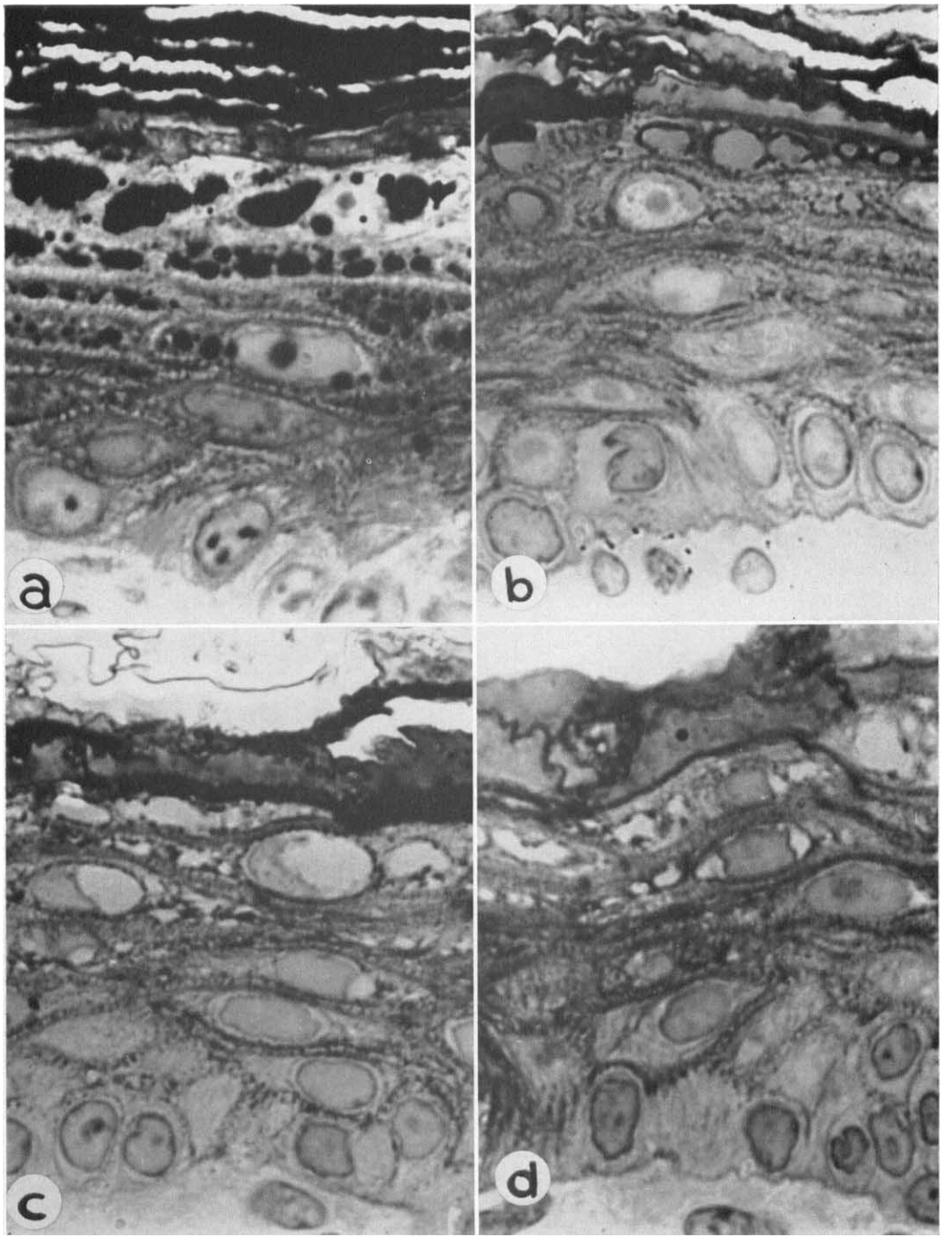

FIG. 1. Non-extracted newborn rat epidermis (a); plastic embedded and toluidine blue stained section shows typical staining of keratohyalin granules. After 2 hours extraction (b) keratohyalin granules show reduced staining. After 6 hours (c) extraction of densely stained material from keratohyalin granules is pronounced, and by 24 hours (d) the material is lost from cornified cells.

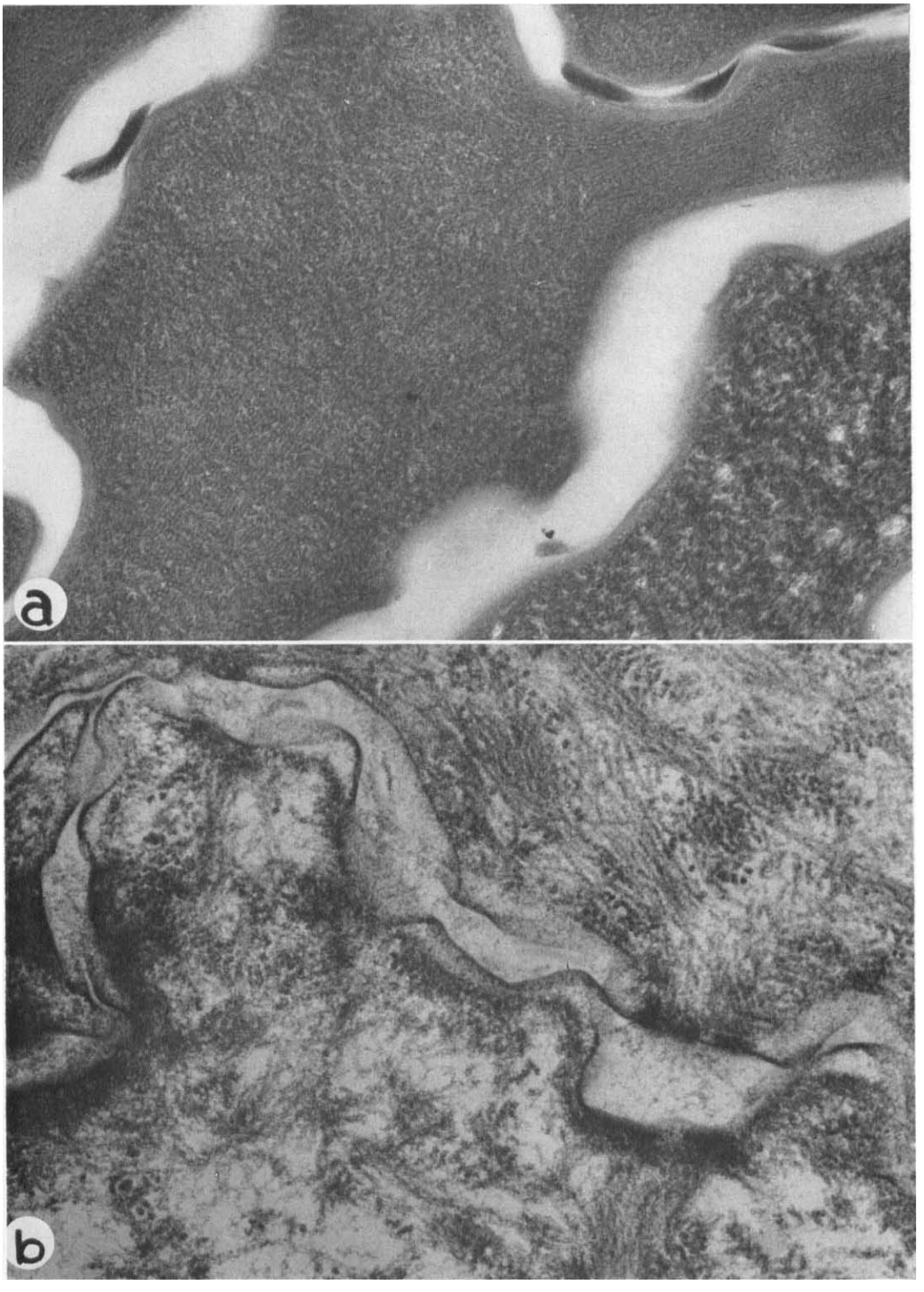

FIG. 2. Non-treated cornified cells (a) showing a typical keratin pattern. After extraction (b), filaments stain densely and many attach to cell membrane at attachment plaques.

FIG. 3. Lower power electron micrograph of the attachment plaques of the desmosomes observed in cornified cells by 24 hours extraction.

concentration of grains over keratohyalin granules was observed after injection of histidine-H^3, but distribution of labels remained essentially the same after injection of leucine-H^3. In extracted epidermis no recognizable changes in distribution of radioactivity were seen after injection of leucine-H^3, but after injection of histidine-H^3 the number of grains appearing in the granular layer was reduced considerably Fig. 6b). Grain counts made in extracted epidermis 30 minutes after injection of histidine-H^3 are summarized in Table II. The number of labels appearing in the basal layer was arbitrarily set as one, and compared to that of the Malpighian and granular layers.

DISCUSSION

The results indicate that certain epidermal proteins can be solubilized in 0.1 N NaOH after use of glutaraldehyde, a fixative widely used for electron microscopy (11). Aldehydes fix by addition, not by coagulation, to "tan" or mechanically stabilize proteins and reduce their ability to bind water. They cross link protein chains, most likely by formation of a methylene bridge linking two side groups of amino acids (12). Experimentally, aldehydes can combine with almost any one of a number of different functional groups of a peptide, but in the pH and temperature range used for tissue fixation (pH 7–8, and 0–4° C), the amino group on a side chain of a peptide, such as found in lysine, seems most reactive and usually starts the reaction. The other side of the cross-link may be provided by other amino groups or by the indol group of tryptophane, the phenyl group of tyrosine, the imidazole group of histidine or by amide groups (7, 12). During fixation the binding capacity of protein to aldehyde is determined by the amino acid sequence and confirmational structure of the protein, for example, polyglycine binds less than one twentieth as much as polyglutamine (7). Thus, it should be possible to selectively extract proteins from glutaraldehyde fixed tissue due to variable solubilities of the proteins composing the tissue.

Matoltsy and Matoltsy have studied the solubilities of different cellular components of

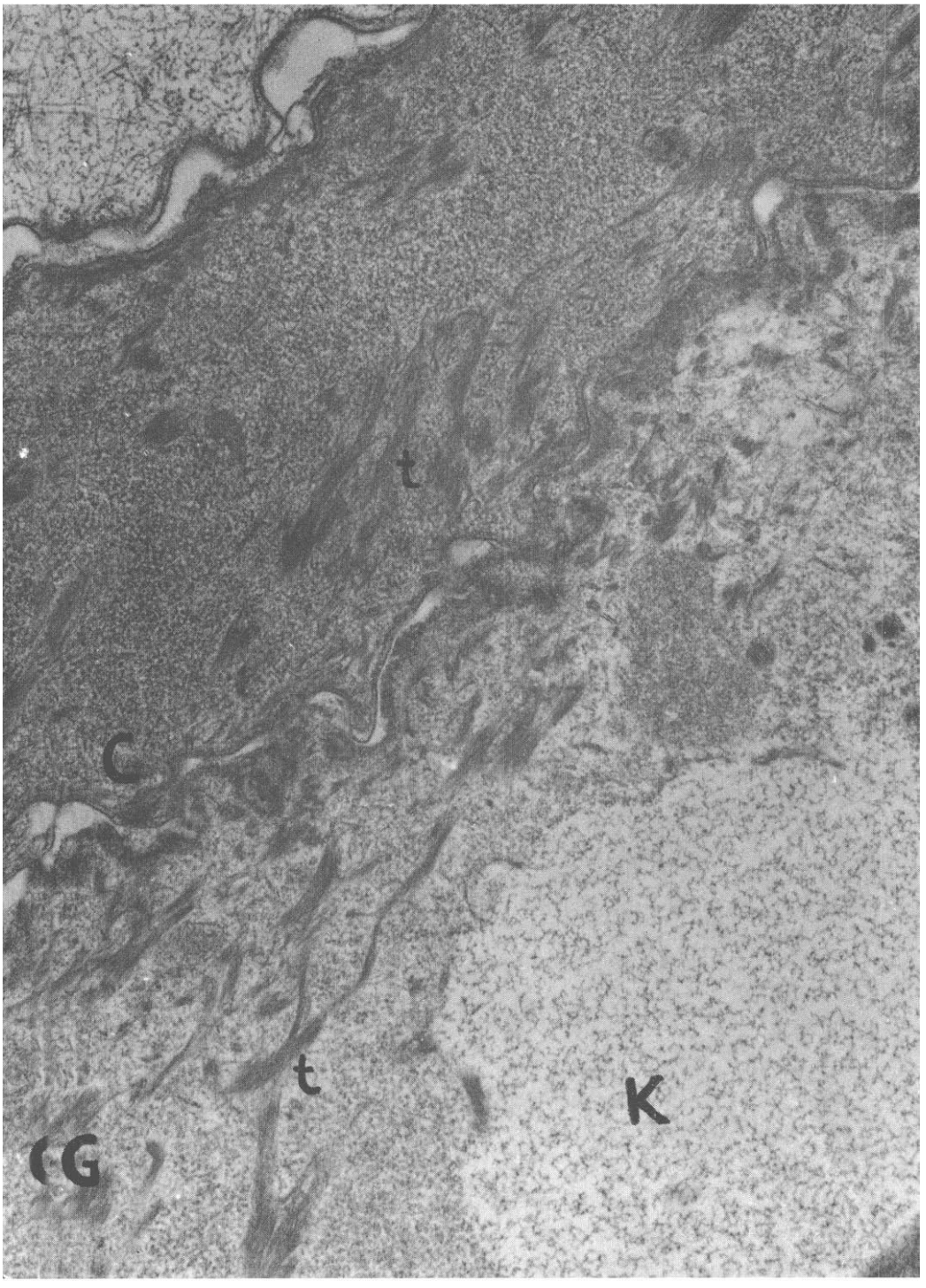

FIG. 4. Cornified cell (C) just above a granular cell (G) demonstrates its internal structure after extraction of densely stained matrix. Tonofibrils (t) which resemble those of granular cells are observed. Extracted keratohyalin granules (K), containing fine fibrils are readily seen.

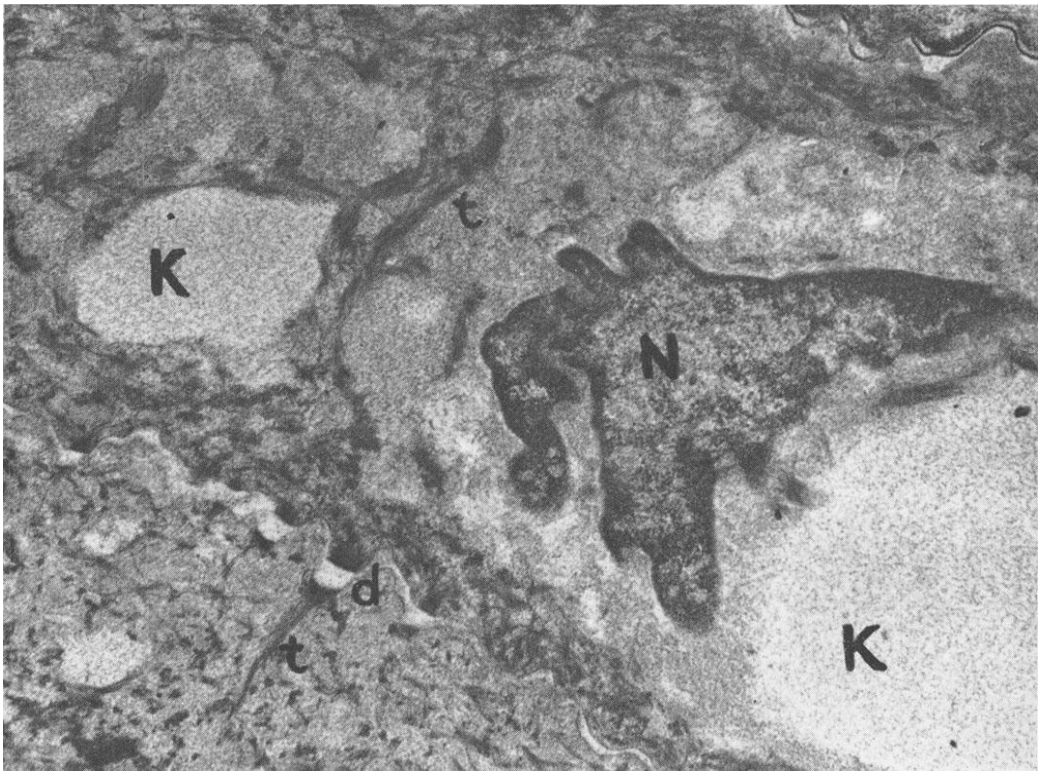

FIG. 5. Granular cells after 24 hours extraction: keratohyalin granules (K), nucleus (N), tonofibrils (t), and desmosomes (d).

TABLE 1

Radioactivity soluble in 0.1 N NaOH sodium hydroxide at various times after extraction

Amino acid	Time after injection	No. of animals	Radioactivity soluble/total (%)		
			2 hour extraction	6 hour extraction	24 hour extraction
Histidine-H^3	30 min	13	5.3 ± 1.2	15.3 ± 0.4	47.0 ± 2.8
	3 hrs	13	8.0 ± 1.6	29.3 ± 3.0	57.0 ± 11.1
	6 hrs	14	13.3 ± 3.0	29.5 ± 0.5	59.0 ± 6.9
Leucine-H^3	30 min	9	2.0 ± 0.2	3.5 ± 0.2	6.9 ± 0.4
	3 hrs	4	---	---	4.5 ± 0.5
	6 hrs	9	2.1 ± 0.2	3.3 ± 0.2	5.0 ± 0.6

fresh epidermis at various pH (13). In alkaline solutions at pH 10.1 and 10.9, epidermal cells including keratohyalin granules dissolved; cornified cells remained intact. Cornified cell components dissolved at pH 11.7, but the plasma membrane remained as an envelope after incubation in 0.1 N NaOH at 5° C for as long as 96 hours (14). Crounse compared the amino acid composition of purified whole callus with that of a protein fraction solubilized by NaOH from the same tissue, and found the overall pattern of amino acid content was quite similar in both samples (15). Alkaline solubilized protein was, therefore, considered to be a major constituent of the whole tissue. The present study shows that fixation modifies the extractability of epidermal components. After fixation significant amounts of radioactivity

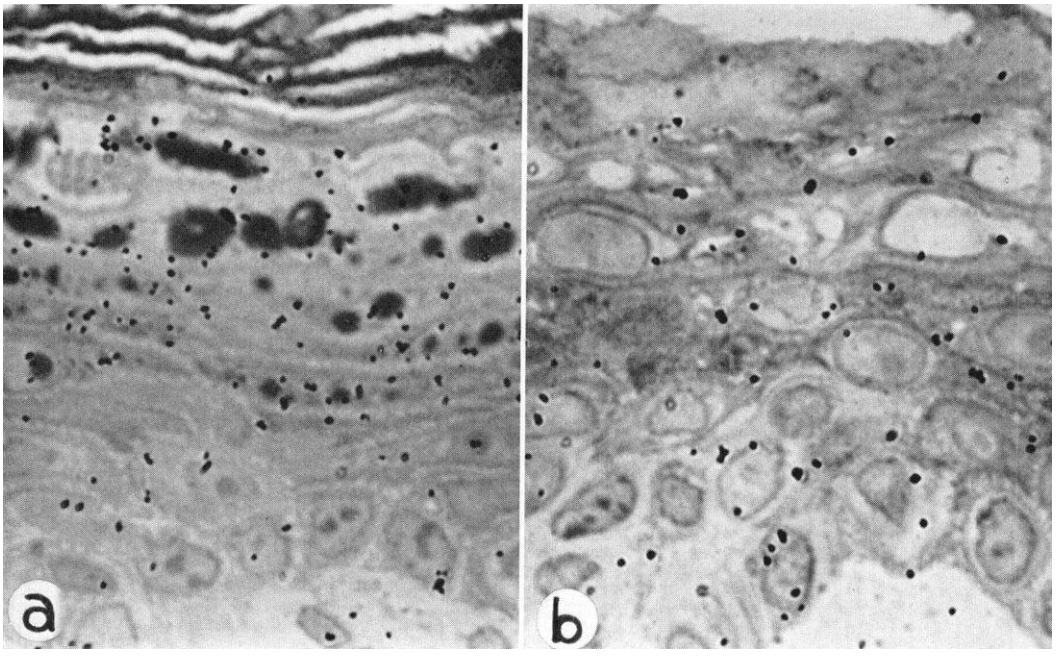

FIG. 6. Autoradiograph demonstrating concentration of silver grains over granular cells (a) 30 minutes after injection of histidine-H^3. After 24 hours extraction (b) the number of silver grains appearing in granular cells is decreased considerably; keratohyalin granules are observed as spaces.

were solubilized from tissue containing histidine-H^3 labeled protein, but when leucine-H^3 was used as a protein-marker, only 5–7 per cent of radioactivity present in the tissue was solubilized. Autoradiographs from tissues similarly treated after injection of histidine-H^3 indicated that the majority of solubilized radioactivity comes from granular cells in which osmiophilic and basophilic materials have disappeared from keratohyalin granules. Protein synthesized in the lower layers appeared to be more reactive to glutaraldehyde and was better "fixed" than that formed in granular cells.

We believe the changes seen in morphology and staining quality of keratohyalin granules after extraction result from loss of histidine-H^3 containing protein and probably other substances such as lipids. Fine fibrillar structures remaining in the area seem to be another constituent of keratohyalin granules. After extraction, cornified cells just above the granular layer reveal a morphology more like granular cells, showing bundles of similar appearing tonofilaments and attachment plaques. In ordinary preparations the dense NaOH extractable substances interfere with observation of the internal structure of these cells, so that the transition from tonofibrils to the finer fibrils of cornified cells is obscured. Extraction provides the opportunity to observe this change.

Ultrastructure of cornified cells has been described in detail (10, 16). They are filled with a mass of low-density filaments embedded in a dense matrix, and present an arrangement called "keratin pattern." Their plasma membranes are thickened and a dense attachment plaque is not seen. After extraction, fibrillar components stain densely and their attachment to the plasma membrane becomes evident, but amorphous substances are removed. These results suggest that the matrix substance behaves very much like the osmiophilic material seen in keratohyalin granules, and supports the idea that substances from keratohyalin granules give rise to the matrix of cornified cells. In addition, it is clear that tonofilament attachments to desmosomes do not disappear in cornified cells, but are masked, and usually are not visible after routine methods of fixation and preparation for electron microscopy.

Brody stated that 85 per cent of cells in the lowermost part of the horny layer showed a

TABLE II

Distribution of silver grains in non-extracted and extracted epidermis 30 minutes after injection of histidine-H³

Extraction of epidermis for 24 hrs. at 37° C	Ratio of grain counts		
	Basal layer	Malpighian layer	Granular layer
Extracted epidermis	1	1.36	1.35
Extracted epidermis	1	1.21	1.50
Extracted epidermis	1	1.45	1.62
Average		1.34 ± 0.10	1.48 ± 0.14
Non-treated epidermis	1	1.34	3.79
Non-treated epidermis	1	1.50	3.09
Non-extracted epidermis	1	1.22	3.25
Non-extracted epidermis	1	1.06	3.01
Non-extracted epidermis	1	1.61	4.52
Non-extracted epidermis	1	1.25	3.71
Average		1.33 ± 0.18	3.56 ± 0.52

"keratin pattern," but in the middle or outer layers a smaller per cent of cells had such a pattern (10). Matoltsy has, therefore, described filaments in horny cells as tightly packed and loosely packed (17). The former implies "keratin pattern" and the latter represents densely stained filaments associated with a smaller amount of amorphous material as observed in the mid horny layers. After extraction, cells in the inner layers demonstrated a positive Schiff reaction containing densely stained filaments without amorphous substances, and in the mid layers, where a weak Schiff reaction was observed, complete dissociation of cell contents occurs. It appears that cells in inner layers contain "fixed" fibrils and "non-fixed" amorphous material but cell components of the middle layers are not "fixed" and thus dissolve in NaOH, as occurs in fresh tissue (14). For this reason it seems unfair to conclude, as Swanbeck and Thyresson did (18), that the "keratin pattern" represents merely an early stage in the continued chemical differentiation of cornified cells as they move to the surface. Alternatively we propose the changes in outer horny cells observed by Brody (10) may represent an artefact of incomplete fixation.

The chemical nature of the two components in cornified cells are not known. Rudall isolated two different proteins: fibrous protein precipitating at pH 5.5 and non-fibrous protein precipitating at pH 4.5 (19). Similar proteins were found by Rothberg using 0.005 M NaOH (20). Crounse, however, obtained an alkaline soluble protein in only one fraction which was precipitated at pH 6.3 under the mildest conditions of extraction. He suggested other fractions, such as those precipitated at pH 4.5 and pH 5.5 may be a derivative of a single unit protein. The present finding may represent a peptide fission product with NaOH splitting off a polypeptide chain from the tertiary protein. It is also possible, however, that one of the heterogeneous proteins present in the epidermis was extracted; most likely an amorphous protein while the fibrillar protein remained. Chemical analysis of extracted materials is in progress.

SUMMARY

The solubility of the various cellular constituents of newborn rat epidermis in 0.1 N NaOH was determined after fixation in 3 per cent glutaraldehyde.

1. Osmiophilic and basophilic substances of keratohyalin granules and densely stained amorphous substances of cornified cells were dissolved.

2. Fibril components in cornified cells were resistant.

3. Radioactivity present in epidermal protein at 30 minutes and 6 hours after injection of histidine-H³ was solubilized to the extent of 45 per cent and 59 per cent, respectively. Autoradiography indicated the solubilized radioactivity likely came from granular cells.

4. Only 5–7 per cent of radioactivity was dissolved from protein synthesized after injection of leucine-H³.

Ultrastructural study of the extracted epidermal cells revealed:

1. A distinct attachment plaque present in cornified cells.

2. Transformation of tonofibrils to the fibrillar component of cornified cells continues in cells located just above the granular layer.

This extractive procedure provides improved resolution of the fibrillar contents of horny cells.

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